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Myosin binding protein-C activates thin filaments and inhibits thick filaments in heart muscle cells

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Myosin binding protein-C (MyBP-C) is a key regulatory protein in heart muscle, and mutations in the MYBPC3 gene are frequently associated with cardiomyopathy. However, the mechanism of action of MyBP-C remains poorly understood, and both activating and inhibitory effects of MyBP-C on contractility have been reported. To clarify the function of the regulatory N-terminal domains of MyBP-C, we determined their effects on the structure of thick (myosin-containing) and thin (actin-containing) filaments in intact sarcomeres of heart muscle. We used fluorescent probes on troponin C in the thin filaments and on myosin regulatory light chain in the thick filaments to monitor structural changes associated with activation of demembrated trabeculae from rat ventricle by the C1mC2 region of rat MyBP-C. C1mC2 induced larger structural changes in thin filaments than calcium activation, and these were still present when active force was blocked with blebbistatin, showing that C1mC2 directly activates the thin filaments. In contrast, structural changes in thick filaments induced by C1mC2 were smaller than those associated with calcium activation and were abolished or reversed by blebbistatin. Low concentrations of C1mC2 did not affect resting force but increased calcium sensitivity and reduced cooperativity of force and structural changes in both thin and thick filaments. These results show that the N-terminal region of MyBP-C stabilizes the ON state of thin filaments and the OFF state of thick filaments and lead to a novel hypothesis for the physiological role of MyBP-C in the regulation of cardiac contractility.

cardiac muscle regulation | myosin-binding protein-C | MYBPC3

Muscle contraction is driven by the relative sliding of the actin-containing thin filaments along the myosin-containing thick filaments arranged in a parallel array in the muscle sarcomere (Fig. 1A). Filament sliding in turn is driven by a structural change in the myosin head domains (Fig. 1B) while they are bound to actin, coupled to the hydrolysis of ATP (1). Contraction of skeletal and cardiac muscle is triggered by calcium binding to troponin in the thin filaments, accompanied by a change in the structure of the thin filaments that permits myosin head binding (2). However, the strength and dynamics of contraction are modulated by posttranslational modifications in other sarcomeric proteins, including the myosin regulatory light chain (RLC) (3), which is part of the myosin head, and myosin binding protein-C (4–6) (MyBP-C) (Fig. 1B). In an emerging concept of thick filament regulation in striated muscle that is analogous to myosin-linked regulation in smooth muscle (7–11), RLC and MyBP-C are thought to modulate contraction by controlling the conformation of the myosin heads.

According to this concept, the thick filament has an OFF state in which the myosin heads are folded back against its surface (Fig. 1B), rendering them unavailable for interaction with actin, and an ON state in which the heads are released from the thick filament surface and made available for actin binding. The physiological and pathological significance of thick filament regulation and its relationship to the well-studied thin filament mechanisms remain poorly understood, but much recent attention has focused on MyBP-C for two main reasons. First, mutations in the cardiac

MYBPC3 gene are commonly associated with hypertrophic cardiomyopathy (12, 13), and this association has driven a wide range of studies at the molecular, cellular, and whole-animal levels aimed at understanding the etiology of MYBPC3-linked disease. Second, although MyBP-C is a constitutive component of the thick filament, there is a large body of evidence that it can also bind the thin filaments (14, 15), raising the possibility that one role of MyBP-C may be to synchronize the regulatory states of the thin and thick filaments (11, 15–17).

MyBP-C is localized to the central region or “C-zone” of each half-thick filament (Fig. 1A), appearing in nine transverse stripes with a 43-nm periodicity closely matching that of the myosin heads (Fig. 1B) (10). MyBP-C has 11 Ig-like or fibronectin-like domains (Fig. 1C) denoted C0–C10, with additional linking sequences, notably the MyBP-C “motif” or “m” domain between C1 and C2 and the proline/alanine-rich (P/A) linker between C0 and C1. The m domain has multiple phosphorylation sites (4–6). Constitutive binding to the thick filament is mediated by interactions of domains C8–C10 with myosin and titin. The C1mC2 region binds to the coiled-coil subfragment-2 (S2) domain of myosin adjacent to the myosin heads, and this interaction is abolished by MyBP-C phosphorylation (5); the C0 domain binds to the RLC in the myosin head itself (18). The N-terminal domains of MyBP-C also bind to actin in a phosphorylation-dependent manner (14, 15) (Fig. 1B), and EM and X-ray studies on intact sarcomeres of skeletal muscle suggest that MyBP-C binds to thin filaments under relaxing conditions (10, 11).

The function of MyBP-C and the mechanisms underlying its modulation in cardiomyopathy remain poorly understood, however. Ablation of MyBP-C in a knockout mouse model leads to a hypertrophic phenotype associated with impaired contractile function (19), but cardiomyocytes isolated from these mice

Significance

Myosin binding protein-C (MyBP-C) is a regulatory protein of heart muscle. Mutations in MyBP-C are frequently associated with heart disease, but the mechanism of action of MyBP-C is poorly understood. By characterizing the effects of its N-terminal domains on the structures of the thin and thick filaments in contracting heart muscle cells, we showed that MyBP-C stabilizes the ON state of thin filaments and the OFF state of thick filaments. The results lead to a model for the control of heart muscle contraction in which the regulatory functions of the thin and thick filaments are coordinated by MyBP-C, providing an integrated framework for the design and development of therapeutic interventions in heart disease.

Author contributions: T.K., M.G., and M.I. designed research; T.K. performed research; Z.Y. and Y.-B.S. contributed new reagents/analytic tools; T.K. and M.I. analyzed data; and T.K., M.G., and M.I. wrote the paper.

The authors declare no conflict of interest.

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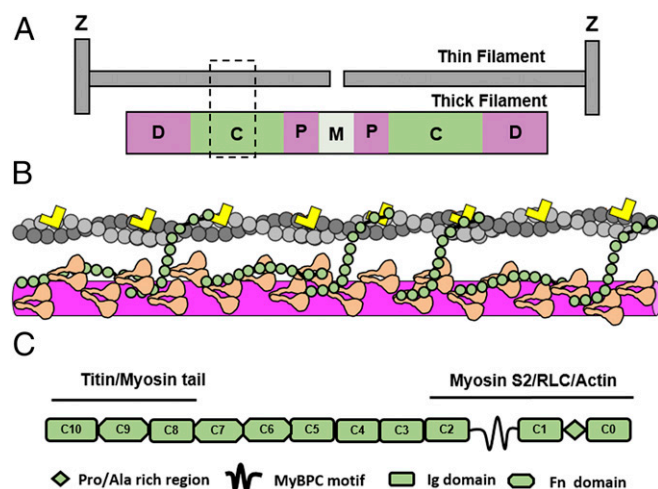


Fig. 1. Sarcomere location and domain architecture of MyBP-C. (A) C-zone (green) of the thick filament in relation to its proximal (P) and distal (D) regions and the thin filament (gray). (B) Cartoon representation of MyBP-C (green) anchored to the thick filament backbone (purple) via its C-terminal domains; myosin heads are pink and troponin is yellow. (C) Domain organization and interactions of MyBP-C.

exhibit increased power output during working contractions (20). A range of studies at the isolated protein and cellular levels have led to the concept that MyBP-C exerts a predominantly inhibitory effect on contractility mediated through two distinct mechanisms (15, 16, 21). MyBP-C may tether myosin heads to the surface of the thick filament, preventing their interaction with actin, and its N terminus may bind to thin filaments, inhibiting interfilament sliding at low load. Other studies, however, have demonstrated an activating effect of MyBP-C mediated by binding of its N-terminal domains to the thin filament. N-terminal fragments of MyBP-C enhance force production in skinned cardiac muscle cells and motility in isolated filament preparations at zero or submaximal calcium concentrations (22–25). The same effect is observed in cardiomyocytes from MyBP-C knockout mice (22), suggesting that the activating effect is not due to competitive removal of an inhibitory effect of native MyBP-C.

To resolve these apparently contradictory hypotheses about the physiological function of the N-terminal domains of MyBP-C, we determined the structural changes in the thick and thin filaments of intact sarcomeres in heart muscle cells induced by N-terminal MyBP-C fragments using bifunctional rhodamine probes on RLC and troponin C (TnC) (26). These probes allowed the structural changes in both types of filament to be directly compared with those associated with calcium activation and myosin head binding in the native environment of the cardiac muscle sarcomere. The results lead to a model for the physiological function of MyBP-C that integrates the regulatory roles of the thin and thick filaments and the inhibitory and activating effects of MyBP-C at the level of the intact sarcomere.

Results

Activation of Rat Ventricular Trabeculae by Rat MyBP-C Fragments. Rat MyBP-C fragments containing the N-terminal sequence from domains C0–C2 (C0C2) activated contraction in skinned ventricular trabeculae of the rat in the absence of calcium (*SI Appendix, Fig. S2A*). A shorter fragment containing only domains C1 and C2 and the intervening m-motif (C1mC2) had a similar effect. The C0C1 fragment, the isolated m-motif, and a C1xC2 construct in which the m-motif was replaced by a flexible linker (GGGGGS)₅, did not activate at concentrations up to 50 $\mu\text{mol/L}$ (*SI Appendix, Figs. S2A and S3*). The C1mC2 construct from the MyBP-C isoform from fast skeletal muscle also did not activate rat trabeculae (*SI Appendix, Fig. S4A*), but a chimera containing

the cardiac C1 and C2 sequences linked by the m-motif from fast skeletal muscle had the same activating effect as cardiac C1mC2 (*SI Appendix, Fig. S4B*). Thus, for rat MyBP-C fragments in rat ventricular trabeculae, the C1mC2 region is sufficient for activation of contraction, and the C0 domain and the P/A linker between C0 and C1 are not required. Tris-phosphorylation of C1mC2 by PKA abolished the activating effect (*SI Appendix, Fig. S2A*). We therefore used the unphosphorylated C1mC2 fragment for all of the experiments described below.

Active isometric force produced by the addition of C1mC2 in relaxing solution had a sigmoidal dependence on C1mC2 concentration, with an EC_{50} of $20 \pm 2 \mu\text{mol/L}$ (mean \pm SEM, $n = 4$) (*SI Appendix, Fig. S2B*). Maximum force was about 60% of that produced by calcium activation at pCa 4.5 in the absence of the fragment (T_0). In the presence of 50 $\mu\text{mol/L}$ C1mC2, isometric force was the same in the absence (pCa 9) and the presence (pCa 4.5) of calcium [$T/T_0 = 56 \pm 4\%$ (mean \pm SEM, $n = 9$) and $56 \pm 6\%$, respectively; *SI Appendix, Fig. S2B*]. Therefore, although 50 $\mu\text{mol/L}$ C1mC2 activates contraction in the absence of calcium, it *inhibits* active force in the presence of calcium. These results are broadly consistent with the reported effects of mouse MyBP-C fragments in skinned rat trabeculae (25), but differ from those of studies using human MyBP-C fragments in human or mouse cardiomyocytes (23) in which the P/A linker region flanked by domains C0 and C1 was required for activation in the absence of calcium. The comparison suggests a functional difference between human and rodent MyBP-C (27) or possibly between cardiomyocytes and trabeculae. The former explanation seems the more likely because rodent and human MyBP-C have roughly 90% sequence identity in the m-motif, but only 36–58% in the P/A linker (28).

C1mC2 Directly Activates Thin Filaments in the Absence of Calcium.

We used site-specific bifunctional rhodamine (BR) probes on TnC to monitor changes in the structure of the thin filaments during activation of cardiac muscle by C1mC2. A BR probe on the C-helix of TnC (BR-TnC-C) was used to monitor the orientation of its N-terminal domain containing the regulatory Ca^{2+} site (29). A probe on the E-helix of TnC (BR-TnC-E) was used to monitor the orientation of the elongated “IT arm” domain of troponin that also contains segments of troponin I (TnI) and troponin T (TnT) (30). The E-helix probe monitors a step in the thin filament signaling pathway that is closely coupled to the azimuthal movement of tropomyosin that uncovers the myosin-binding sites on actin during Ca^{2+} -mediated activation (29).

BR-TnC-C or BR-TnC-E was introduced into skinned ventricular trabeculae to replace most of the native TnC, and the orientation of each probe was measured by polarized fluorescence (26) (*SI Appendix, Table S1*). The peak angle θ_{ME} and SD σ_{ME} of the probe orientation distribution with respect to the filament or trabecular axis under standard relaxing and activating conditions (*SI Appendix, Table S2*) were similar to values reported previously (26). When trabeculae were activated by 50 $\mu\text{mol/L}$ C1mC2 in the absence of Ca^{2+} , θ_{ME} for the TnC C-helix probe (BR-TnC-C) increased over a period of 10–20 min as C1mC2 diffused into the trabeculae and active force developed (*SI Appendix, Fig. S5*). The increase in θ_{ME} was $81 \pm 4\%$ (mean \pm SEM; $n = 5$) of the difference between its values in standard relaxing and activating solutions (Fig. 2A, green and red dashed lines), slightly larger than the corresponding fraction for active force in these experiments (T/T_0 , $63 \pm 8\%$). Transfer to normal activating solution in the presence of C1mC2 (ACT C1mC2) produced a further small increase in θ_{ME} without a further increase in force (T/T_0 was $59 \pm 8\%$). All these changes were fully reversed by sequential washout of C1mC2 and calcium (*SI Appendix, Fig. S5*). The orientation changes of the BR-TnC-C probe induced by addition of C1mC2 were almost independent of the presence of 25 $\mu\text{mol/L}$ blebbistatin (Fig. 2A; *SI Appendix, Table S2*), which completely abolished active force (*SI Appendix, Fig. S5*). Thus, the changes in TnC orientation induced by C1mC2 are not mediated by force-generating myosin heads,

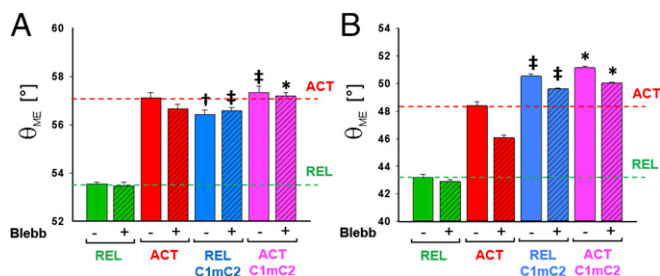


Fig. 2. Orientation (θ_{ME}) of BR-TnC-C (A) and BR-TnC-E (B) probes in relaxing (REL) and activating solution (ACT) and the effects of 50 $\mu\text{mol/L}$ C1mC2 and 25 $\mu\text{mol/L}$ blebbistatin. Means \pm SEM ($n = 5-7$). Statistical significance of differences between values was assessed using a two-tailed paired Student's t test: * $P < 0.05$; † $P < 0.01$; ‡ $P < 0.001$.

suggesting that C1mC2 has a direct effect on the structure of the thin filament that mimics that produced by binding of Ca^{2+} .

Similar results were obtained with the BR-TnC-E probe on the IT arm of troponin (Fig. 2B; *SI Appendix*, Table S2), except that the orientation changes induced by 50 $\mu\text{mol/L}$ C1mC2 were larger than those produced by maximal calcium activation. Slightly smaller increases in θ_{ME} for the E-helix probe were observed in the presence of blebbistatin (Fig. 2B), but the change in θ_{ME} induced by C1mC2 in the presence of blebbistatin was more than twice as large as that produced by calcium activation under the same conditions.

C1mC2 Induced Changes in Myosin RLC Orientation. To investigate the role of structural changes in the thick filaments during activation by C1mC2, we used bifunctional sulphorhodamine (BSR) probes on the RLC region of myosin, either cross-linking RLC helices B and C (BSR-RLC-BC), roughly parallel to the short hook helix of the myosin heavy chain at the C terminus of the head domain, or along its E-helix (BSR-RLC-E), roughly parallel to the lever arm of the head.

When trabeculae were activated by 50 $\mu\text{mol/L}$ C1mC2 in the absence of calcium, θ_{ME} for both BSR-RLC-BC (Fig. 3A; *SI Appendix*, Fig. S6) and BSR-RLC-E (Fig. 3B) changed from their respective relaxed values (green dashed lines) toward values for calcium activation (red dashed lines). The average changes in θ_{ME} induced by 50 $\mu\text{mol/L}$ C1mC2 were $63 \pm 4\%$ and $43 \pm 11\%$ (mean \pm SEM; $n = 5-7$) of those associated with maximum calcium activation for BSR-RLC-BC and BSR-RLC-E, respectively (*SI Appendix*, Table S2), which is close to the corresponding fractions for active force of $52 \pm 4\%$ and $42 \pm 3\%$. No further changes in θ_{ME} were produced by calcium activation in the presence of 50 $\mu\text{mol/L}$ C1mC2 (*SI Appendix*, Fig. S7).

Blebbistatin had a direct effect on the structure of the thick filament in the absence of calcium (Fig. 3; *SI Appendix*, Table S2), signaled by a change in θ_{ME} for both BSR-RLC-BC and BSR-RLC-E from the values in standard relaxing solution in the opposite direction to that produced by calcium activation. This effect of blebbistatin on thick filament structure is consistent with previous results from skeletal muscle showing that it stabilizes the switch-2 closed state of the myosin head and the helical order of myosin filaments (31, 32) associated with the asymmetric “J-motif” seen in electron-microscopy reconstructions of isolated thick filaments from both skeletal and cardiac muscles (8, 33). This more ordered state of the thick filament is expected to inhibit the interaction of the myosin heads with thin filaments; i.e., it is a more OFF state of the thick filament.

Addition of calcium in the presence of blebbistatin produced no significant change in θ_{ME} for the RLC probes (Fig. 3; *SI Appendix*, Table S2), suggesting that the thick filaments remain in the OFF state. Addition of C1mC2 in the presence of blebbistatin produced no significant change in θ_{ME} for BSR-RLC-E (Fig. 3B), but a further small increase in θ_{ME} for BSR-RLC-BC (Fig. 3A), i.e., a further change in RLC orientation in the direction

associated with the OFF state of the thick filament. These RLC probe experiments therefore suggest that C1mC2, like blebbistatin, stabilizes the OFF structure of the thick filament and inhibits contractility, in contrast to the activating effect of C1mC2 on the structure of the thin filament.

Addition of 50 $\mu\text{mol/L}$ tris-phosphorylated C1mC2 in relaxing solution had no effect on the orientation of either the BSR-RLC-BC or the BR-TnC-E probe (*SI Appendix*, Fig. S8), as expected from the absence of force activation by this fragment.

Low Concentrations of C1mC2 Alter the Calcium Dependence of Thin Filament Activation.

Concentrations of N-terminal fragments of MyBP-C that are too low to activate cardiac muscle in the absence of calcium increase the sensitivity and decrease the cooperativity of the force–calcium relationship (23, 25, 34). We used the TnC E-helix probe to investigate the structural basis of these effects in the presence of 2 $\mu\text{mol/L}$ C1mC2, a concentration that did not alter active force in either relaxing or activating solution. This concentration of C1mC2 increased the $p\text{Ca}$ for half-maximal force, $p\text{Ca}_{50}$, by about 0.25 $p\text{Ca}$ units (Fig. 4A; *SI Appendix*, Table S3), indicating a significant increase in calcium sensitivity, and the steepness of the force– $p\text{Ca}$ relationship, described by the Hill coefficient (n_H), decreased from 5.0 ± 0.2 to 2.4 ± 0.2 . The net effect is a large increase in active force at calcium concentrations in the physiological range; at $p\text{Ca}$ 5.7, for example, active force increased from 3 to 50% of its maximum value. C1mC2 (2 $\mu\text{mol/L}$) had no effect on the orientation of the E-helix of TnC in the absence of calcium (Fig. 4B), but significantly increased θ_{ME} for the BR-TnC E-helix probe for all calcium concentrations at which active force was generated. The increase in $p\text{Ca}_{50}$ and the decrease in the Hill coefficient (n_H) of θ_{ME} for BR-TnC-E induced by 2 $\mu\text{mol/L}$ C1mC2 were similar to those for active force (*SI Appendix*, Table S3).

When active force was abolished by 25 $\mu\text{mol/L}$ blebbistatin, the increase in θ_{ME} for BR-TnC-E induced by calcium was reduced at all calcium concentrations (Fig. 4C, filled circles). In addition, the calcium sensitivity of θ_{ME} decreased (as measured by $p\text{Ca}_{50}$) (*SI Appendix*, Table S3), and the steepness of the calcium dependence, measured by the Hill coefficient n_H , also decreased. Addition of 2 $\mu\text{mol/L}$ C1mC2 in the presence of blebbistatin (Fig. 4C, open circles) reversed the decrease in θ_{ME} produced by blebbistatin at high $[\text{Ca}^{2+}]$ and increased $p\text{Ca}_{50}$ by 0.22 $p\text{Ca}$ units (Fig. 4C; *SI Appendix*, Table S3). However, the steepness of the relationship between θ_{ME} and $[\text{Ca}^{2+}]$ remained low; in the presence of 2 $\mu\text{mol/L}$ C1mC2, n_H was about 2.5 and roughly independent of force inhibition by blebbistatin. The interpretation of these results is considered in *Discussion*.

Low Concentrations of C1mC2 Alter the Calcium Dependence of Thick Filament Activation.

The calcium dependence of the orientation of RLC probes in cardiac trabeculae has not been reported previously. In the absence of C1mC2, θ_{ME} for the BSR-RLC-BC

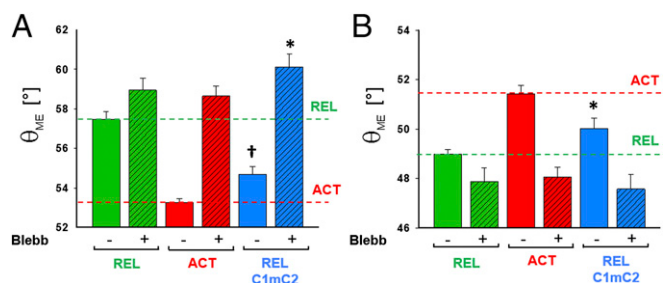


Fig. 3. Orientation (θ_{ME}) of BSR-RLC-BC (A) and BSR-RLC-E (B) probes in relaxing (REL) and activating solution (ACT) and the effects of 50 $\mu\text{mol/L}$ C1mC2 and 25 $\mu\text{mol/L}$ blebbistatin. Means \pm SEM ($n = 5-7$). Statistical significance of differences between values was assessed using a two-tailed paired Student's t test: * $P < 0.05$; † $P < 0.01$.

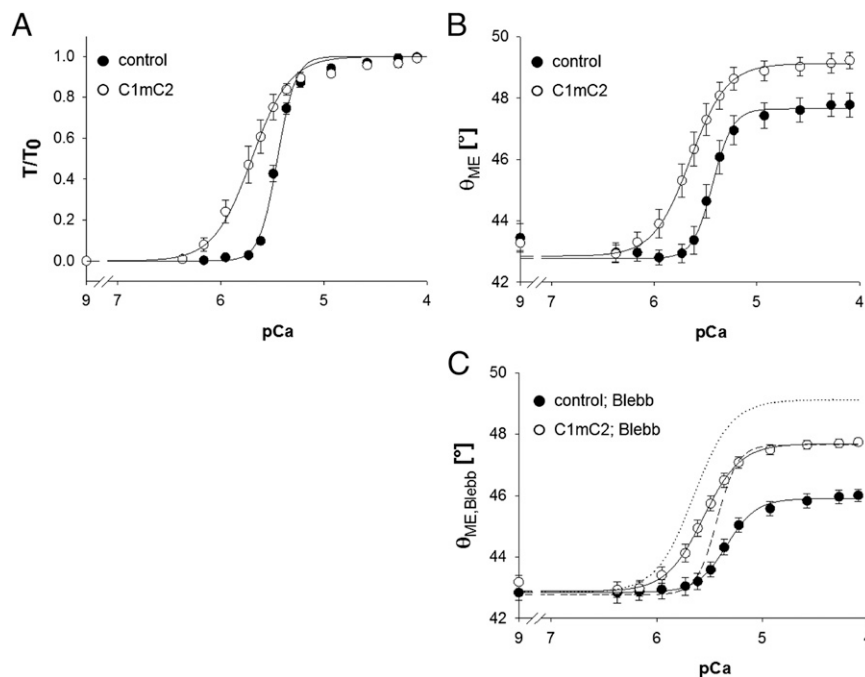


Fig. 4. Calcium dependence of force and TnC E-helix probe orientation in the absence (filled circles) and presence (open circles) of 2 $\mu\text{mol/L}$ C1mC2. (A) Force-pCa relation. (B and C): θ_{ME} for BR-TnC-E in the absence (B) and the presence (C) of 25 $\mu\text{mol/L}$ blebbistatin. Means \pm SEM ($n = 5$).

probe showed a biphasic dependence on calcium concentration (Fig. 5B, filled circles), increasing between pCa 9 and 6 before decreasing steeply between pCa 6 and 5.4, with $n_H = 8.4 \pm 1.0$ in this range (SI Appendix, Table S3), compared with 5.5 ± 1.0 for force. The origin of the change in RLC orientation in the pCa range 9–6 is unknown.

Addition of 2 $\mu\text{mol/L}$ C1mC2 increased pCa_{50} for force by 0.28 ± 0.02 pCa units (mean \pm SE, $n = 5$) and decreased n_H for force to 3.0 ± 0.2 in these trabeculae (Fig. 5A), similar to the results described above for the TnC E-helix probe. It also abolished the biphasic response of θ_{ME} for the BSR-RLC-BC probe (Fig. 5B, open circles), so that the θ_{ME} data for this probe were well described by the Hill equation with $pCa_{50} = 5.97 \pm 0.03$ and $n_H = 3.2 \pm 0.2$ (SI Appendix, Table S3). pCa_{50} for θ_{ME} remained significantly higher than that for force, but the steepness of the θ_{ME} -pCa relationship was similar to that of force in the presence of 2 $\mu\text{mol/L}$ C1mC2, with n_H close to 3, as reported above for the TnC probes.

Discussion

MyBP-C C1mC2 Directly Activates the Thin Filament. The results from the TnC probes reported above provide strong evidence that N-terminal domains of MyBP-C bind to thin filaments in the intact sarcomere of a heart muscle cell and induce a structural change that mimics or exceeds that associated with normal calcium activation. The stabilization of the ON structure of the thin filament induced by 50 $\mu\text{mol/L}$ C1mC2 in the absence of calcium was still present when active force generation was inhibited by blebbistatin. The latter observation makes it highly unlikely that the activating effect of C1mC2 on the thin filament is a secondary effect of binding of myosin heads to the thin filament. Strong binding of myosin heads to thin filaments is blocked by blebbistatin; and weak binding, which may not be blocked by blebbistatin, is almost independent of calcium and does not activate the thin filament (35). Moreover, the amplitude and kinetics of the calcium-induced changes in the orientation of the analogous C- and E-helix probes on TnC in skeletal muscle when active force is inhibited by N-benzyl-p-toluenesulphonamide, which has similar effects to blebbistatin, are the same as those produced when myosin binding to actin is completely abolished by removing overlap between thin

and thick filaments (29). We conclude that the results of the blebbistatin experiments reported here exclude the possibility that changes in thin filament structure induced by C1mC2 are a secondary consequence of its effects on the thick filament.

These effects of C1mC2 on thin filament structure in the absence of calcium are of the same size as those produced by binding of myosin heads in the absence of MgATP (in rigor), as reported by the same TnC probes. The average increases in θ_{ME} for BR-TnC-C and BR-TnC-E induced by 50 $\mu\text{mol/L}$ C1mC2 were 2.8° and 7.3° , respectively (SI Appendix, Table S2), which are very close to the corresponding values of the analogous parameter θ_r for the changes induced by myosin heads binding in rigor in the absence of calcium, 2.5° and 8.1° , respectively (26). The comparison suggests that in the intact sarcomere C1mC2, like myosin heads in rigor, can displace tropomyosin azimuthally around the thin filament toward the ON position in which it would not interfere with myosin binding, as observed in isolated thin filaments (36).

A much lower concentration of C1mC2, 2 $\mu\text{mol/L}$, was sufficient to increase the level of thin filament activation significantly at calcium concentrations in the physiological range (Fig. 4). The activating effects of 2 $\mu\text{mol/L}$ C1mC2 and calcium are synergistic; the N-terminal region of MyBP-C renders the thin filament more sensitive to calcium, and this leads to a large increase in active force around pCa 5.8. The effects of low concentrations of

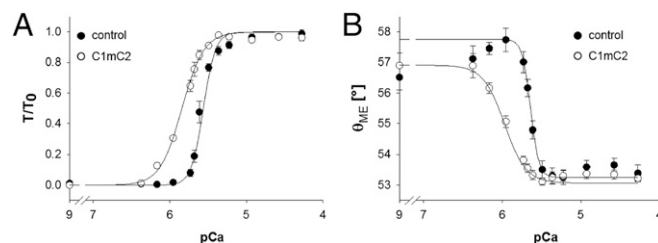


Fig. 5. Calcium dependence of force and RLC BC-helix probe orientation in the absence (filled circles) and the presence (open circles) of 2 $\mu\text{mol/L}$ C1mC2. (A) Force-pCa relation. (B) θ_{ME} for BSR-RLC-BC. Means \pm SEM ($n = 5$).

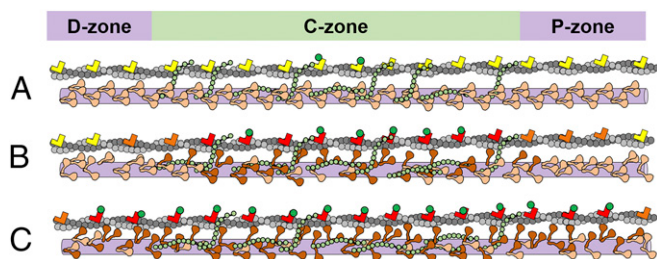


Fig. 6. Proposed role of MyBP-C in cardiac muscle regulation. (A–C) Progressively increasing calcium concentrations, corresponding to pCa 6.0, 5.8, and 5.4 under the conditions of our experiments. Colors are as in Fig. 1B except that orange and red troponins denote increased activation state, calcium is green, and myosin heads are light brown in the ordered OFF state and dark brown in the ON state.

C1mC2 were still present when active force was abolished by blebbistatin, showing that they are not mediated by binding of myosin heads; the synergistic effects of C1mC2 and calcium operate at the level of the thin filament. Low concentrations of C1mC2 also reduced the steepness of the calcium dependence at the level of both the orientation change of the TnC E-helix and the active force (Fig. 4; *SI Appendix, Table S3*); the Hill coefficient n_H was about 2.5, independent of the presence of blebbistatin. Thus, C1mC2, like blebbistatin, reduces the cooperativity of calcium activation, suggesting that the effects of both C1mC2 and blebbistatin are mediated by stabilization of the OFF structure of the thick filament (31). The residual cooperativity of calcium activation under these conditions, corresponding to an n_H of around 2.5, is likely to be an intrinsic property of the thin filament (26, 37), whereas the higher n_H value around 5 under control conditions may be due to the additional contribution of thick filament-based cooperativity, as discussed below.

MyBP-C C1mC2 Inhibits the Thick Filament. At calcium concentrations producing maximal activation, isometric force in the presence of 50 $\mu\text{mol/L}$ C1mC2 was about 60% of that in its absence. C1mC2 therefore inhibits active force under these conditions, despite its activating effect on the structure of the thin filament. The comparison suggests that the inhibitory effect of C1mC2 is mediated by a direct effect of C1mC2 on myosin and the thick filament. This conclusion is supported by the finding that changes in the orientation of the RLC probes induced by 50 $\mu\text{mol/L}$ C1mC2 in either the absence or the presence of calcium were about 60% of those produced by normal calcium activation (Fig. 3; *SI Appendix, Fig. S7*), consistent with the 60% active force produced under these conditions, but less than the corresponding percentage for the orientation change of the TnC probes (Fig. 2). Moreover, the changes in the orientation of the RLC probes induced by C1mC2 were either abolished or reversed by blebbistatin (Fig. 3), again in marked contrast to the results from the TnC probes. Taken together, these results suggest that C1mC2 inhibits contractility by stabilizing the OFF structure of the thick filament, although an alternative inhibitory mechanism involving direct competition between C1mC2 and myosin for actin-binding sites cannot be excluded by the present results.

A much lower concentration of C1mC2, 2 $\mu\text{mol/L}$, did not inhibit force or the orientation change of the RLC BC-helix probe at maximal $[\text{Ca}^{2+}]$, but decreased the steepness of the calcium dependence of the orientation change. The Hill coefficient (n_H) for the RLC orientation change was about 3, similar to that of BR-TnC-E and of active force under the same conditions. The abolition of the biphasic calcium dependence of BSR-RLC-BC orientation and of its very steep slope in the range pCa 6.0–5.4 (Fig. 5) by this low concentration of C1mC2 suggests that these effects may be mediated by native MyBP-C molecules in the C-zone, as discussed further below.

Inferring the Function of Native MyBP-C from the Effects of MyBP-C Fragments. The use of MyBP-C fragments as a model system to investigate the function of native MyBP-C has significant limitations. Native MyBP-C is confined to nine stripes at 43-nm intervals in the C-zone in the central third of each half-thick filament (Fig. 14). Within the C-zone, there are three molecules of MyBP-C per 43-nm length of thick filament (38), corresponding to a 1:6 ratio of MyBP-C to myosin heads, or roughly 1:12 MyBP-C:actin monomers. MyBP-C fragments are not subject to these stoichiometric limitations and could in principle bind to every actin or every myosin molecule in the sarcomere.

The concentrations of C1mC2 used in the present experiments are similar to or lower than the native concentrations in skinned trabeculae. The d_{10} spacing of the thick filament lattice planes is about 46 nm in this preparation (39), so the volume of a unit cell corresponding to the 43-nm repeat of a thick filament is $43 \times 46 \times 46 \times 2/\sqrt{3} \text{ nm}^3$, giving a local MyBP-C concentration of $\sim 50 \mu\text{mol/L}$. This is higher than the C1mC2 concentration required for half-maximum activation in the absence of calcium, 20 $\mu\text{mol/L}$, and much higher than the 2 $\mu\text{mol/L}$ that produced substantial effects on thin and thick filament structure at physiological calcium concentrations (Figs. 4 and 5). The effects of competition between exogenous MyBP-C fragments and native full-length MyBP-C are likely to be small in these experiments for two reasons: the high stoichiometry of exogenous fragments to native MyBP-C and the fact that the native MyBP-C is phosphorylated at $\sim 2 \text{ mol Pi/mol MyBP-C}$ under the conditions of the present experiments (*SI Appendix, Fig. S1*), which is expected to inhibit its interactions with both myosin S2 and actin (5, 14). The finding that the effects of exogenous C1mC2 fragments are still present in MyBP-C knockout mice (22) also argues strongly against a significant role for competition with native MyBP-C.

The present results provide further evidence that the MyBP-C fragments are a useful model for studying the function of the full-length protein, particularly in the case of the activating effect on thin filaments, which has been controversial (15). The fragments have substantial activating effects at a concentration of 2 $\mu\text{mol/L}$ (Fig. 4), much lower than the native concentration of MyBP-C in the C-zone, and activating effects dominate inhibitory effects under those conditions (Figs. 4 and 5). The similarity of the structural changes in the thin filament produced by C1mC2, by calcium and by binding of myosin heads also argues against the activating effect being purely an artifact, as do its abolition by phosphorylation and the sequence and species specificity of the effects (22, 25, 27).

Role of MyBP-C in the Regulation of Cardiac Contractility. The above results, together with those of a wide range of previous studies referenced in the Introduction and recent reviews (15, 16, 21), suggest the following working hypotheses for the function of native MyBP-C:

- i) The N-terminal region of MyBP-C binds to thin and thick filaments with about the same affinity (10, 14).
- ii) Binding of the N-terminal region of MyBP-C to thin filaments stabilizes their ON state.
- iii) Binding of the N-terminal region of MyBP-C to thick filaments stabilizes their ordered OFF state.
- iv) These effects are transmitted along both thin and thick filaments by cooperative transitions in filament structure.

In relation to number iv, the concept of cooperative transmission of activation state along thin filaments, mediated by end-to-end interactions between tropomyosins in adjacent regulatory units, is well established (2). Cooperative structural transitions in the thick filaments from both skeletal and cardiac muscle are implied by intermolecular interactions between myosin heads in the OFF state (8, 9, 33).

According to this working hypothesis, the structures of the thin and thick filaments at diastole might be as diagrammed in Fig. 6A. Some of the MyBP-C molecules (green) in the C-zone are

bound to myosin S2 via their N-terminal domains, stabilizing the ordered OFF state of the thick filaments in which myosin heads (light brown) are folded back against the filament surface. The N-terminal regions of other MyBP-Cs are bound to thin filaments (gray), increasing the calcium sensitivity of regulatory units in the C-zone. However, the whole thin filament remains OFF, as denoted by the yellow troponins, as a result of the dominant effect of the roughly three-quarters of regulatory units outside the C-zone that are not calcium-sensitized. The whole thick filament is also OFF. At a slightly higher Ca^{2+} concentration (Fig. 6B), the region of the thin filament opposite the C-zone starts to become activated whereas the rest of the thin filament remains OFF. As a result, myosin heads in the C-zone bind to actin, and fewer MyBP-Cs may be bound to the thin filament as a result of displacement by myosin heads or because tropomyosin competes with their actin-binding sites. A combination of structural changes in MyBP-C and myosin heads in the C-zone leads to local activation of the thick filament as myosin heads leave the folded OFF state, and this structural change is propagated along the thick filament by its longitudinal cooperativity. As $[\text{Ca}^{2+}]$ increases further (Fig. 6C), these effects are amplified, and the P- and D-zones of the sarcomere are progressively recruited and contribute to maximal active force. However, the inhibitory effect of MyBP-C on the thick filament is still present (*SI Appendix, Fig. S7*), allowing the ON state of the thick filament to be modulated by physiological control of the level of MyBP-C phosphorylation at levels intermediate between the tris-phosphorylated state at which its inhibition is undetectable and the dephosphorylated state at which it is maximal.

According to this hypothesis, one function of the C-zone may be to enable graded rather than uniform activation of the muscle sarcomere; myosin heads may be recruited at higher $[\text{Ca}^{2+}]$ because the length of the active region of the filaments increases. This effect might contribute to length-dependent regulation and the Frank-Starling relation (15). The C-zone may also act as a sarcomeric signaling element that transmits the activation signal from the thin to the thick filaments, with cooperativity in both the thin and thick filaments subsequently transmitting the activation signal along the sarcomere from the C- to the P- and D-zones. Finally, thick filament cooperativity could make a contribution to the steep $[\text{Ca}^{2+}]$ dependence of contractility similar to that of the thin filaments. In this holistic view of the regulation of cardiac contractility, the effective regulatory unit in heart muscle is the sarcomere itself, and mutations in the sarcomeric proteins linked to hypertrophic cardiomyopathy have a common mechanistic target, providing an integrated framework for the design and development of therapeutic interventions in heart disease.

Materials and Methods

Protein production, preparation of cardiac trabeculae, protein exchange protocols, and fluorescence polarization experiments were performed according to routine protocols published elsewhere. Details of materials and methods are provided in *SI Appendix*.

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